

IN THE SPECIFICATION:

Please replace the paragraph beginning on Page 1, line 6, and ending on Page 1, line 11, with the following:

The present invention relates to a megakaryocyte differentiation factor, gene coding for the factor and a process for production thereof. The megakaryocyte differentiation factor is useful as a hemopoietic stimulating factor for megakaryocyte-platelet ~~linease~~ lineage.

Please replace the paragraph beginning at Page 5, line 1, and ending on Page 5, line 2, with the following:

Fig. 6 shows a result of an ~~iseleetroie~~ isoelectric focusing of the purified megakaryocyte differentiation factor.

Please replace the paragraph beginning at Page 5, line 12, and ending on Page 5, line 17, with the following:

Fig. 8 is a graph comparing acetylcholine esterase activity of megakaryocytes derived from mouse bone marrow cells cultured for 5 days in the presence or absence of purified megakaryocyte differentiation factor (55 kDa protein) and with or without addition of ~~IL-6~~ IL-3.

Please replace the paragraph beginning at Page 5, line 36, and ending on Page 6, line 1, with the following:

Fig. 12 is a graph showing an expression of megakaryocyte differentiation factor (TP55) in hemolymph of Bombyx mori after ~~reparation separation~~ by Matrex Blue A column chromatography.

Please replace the paragraph beginning at Page 6, line 9, and ending on Page 6, line 25, with the following:

In addition to the above-defined megakaryocyte differentiation factor, the present invention relates to megakaryocyte differentiation factors obtainable from transformants such as cells or animals constructed by gene technology and having the same amino acid sequence as the above-defined megakaryocyte differentiation factor, an amino acid sequence wherein a portion of the above-defined megakaryocyte differentiation factor is deleted, an amino acid sequence wherein a portion of the above-defined megakaryocyte differentiation factor is replaced with other amino acid or amino acid sequence, or an amino acid sequence wherein one or more than one amino acid sequence is added to the above-defined megakaryocyte differentiation factor, or having an amino acid sequence including a combination of said modifications.

Please replace the paragraph beginning at Page 6, line 26, and ending on Page 6, line 37, with the following:

Moreover, the present invention relates to megakaryocyte differentiation factor having the amino acid sequence shown in SEQ ID NO: 30, an amino acid sequence wherein a portion of the amino acid sequence shown in SEQ ID NO: 30 is deleted, an amino acid sequence wherein a portion of the amino acid sequence shown in SEQ ID NO: 30 is replaced with ~~an other~~ another amino acid or amino acid sequence, or an amino acid sequence wherein one or more than one amino acid sequence is added to the amino acid sequence shown in SEQ ID NO: 30, or having an amino acid sequence including a combination of said modifications.

Please replace the paragraph beginning at Page 7, line 19, and ending on Page 7, line 21, with the following:

The above-mentioned various ~~modification~~ modifications can be carried out by a conventional technique such as site-specific ~~a~~ mutagenesis mutagenesis.

Please replace the paragraph beginning at Page 7, line 22, and ending on Page 8, line 1, with the following:

The number of amino acids involved in the modification, such as addition, deletion or replacement, is not limited, but as for addition the number of amino acid depends on the number of amino acids, for example, that of the functional peptide used in a hybrid protein with the megakaryocyte differentiation factor of the present invention or that of a signal peptide added to the present factor, namely and depends on the purpose of the

modification. For deletion, ~~as for deletion~~ the number of amino acids may be designed or determined so as to maintain megakaryocyte differentiation activity and it is, for example, 1 to 30, preferably 1 to 20 or it can be that of region other than the active region of the present factor. For replacement, ~~and as for replacement~~ the number of amino acids also may be designed or determined so as to maintain megakaryocyte differentiation activity and it is, for example, 1 to 10, preferably, 1 to 5.

Please replace the paragraph beginning at Page 8, line 2, and ending on Page 8, line 10, with the following:

An addition or improvement of ~~an a~~ signal sequence, choice of host-vector system, and improvement of expression regulatory region may provide efficient expression. In addition, a host may be chosen to provide a glycosylated product. Moreover, a polynucleotide coding for at least one of the amino acid sequences shown in SEQ ID NO: 1 to 9 may be used as a DNA probe for cloning a gene.

Please replace the paragraph beginning at Page 8, line 35, and ending on Page 9, line 10, with the following:

To assay a megakaryocyte differentiation factor, megakaryocyte-series cell lines (for example, CMK cells or cells derived therefrom), or mouse bone marrow cells may be used. For example, activity of acetylcholine esterase which is known to be specifically detected in murine megakaryocytes is carried out using mouse bone marrow cells according

to the Ishibashi et al. method (Ishibashi, T. et al., Proc. Natl. Acad. Sci. USA 86, 5953, 1989). In addition, histochemical detection of megakaryocytes is carried out by subjecting cultured bone marrow cells to acetylcholine esterase staining and May-Gruenwald-Giemsa's Giemsa's staining and the observing the shape of the stained cells.

Please replace the paragraph beginning at Page 13, line 23, and ending on Page 13, line 32, with the following:

As expression vectors, plasmid, phage, phagemid, virus such as baculo virus, vaccinia virus or the like can be used. A promoter in an expression vector is selected depending on host used. For example, lac promoter, trp promoter and the like can be used as bacterial promoters, and adhl promoter, pck promoter and the like can be used as yeast promoters. On the other hand, ~~baeuro-virus~~ baculovirus polyhedrin promoter can be used as insect promoter, and Simian virus 40 early or late promoter can be used for animal cells.

Please replace the paragraph beginning at Page 15, line 13, and ending on Page 15, line 31, with the following:

Myeloid cells were pushed out of the femur of female BDF₁ mouse and suspended in an α -MEM medium (Flow Laboratories, Inc. McLean, VA, USA). Percoll layers having different densities (Pharmacia LKB Biotechnology, Tokyo) were overlaid overlaid, and the bone marrow cell suspension was put thereon, followed by centrifugation at

400 x g for 20 minutes. Mononuclear cells collected at the interface of a layer having a density of 1.07 g/ml and a layer having a density of 1.08 g/ml were recovered and washed once with α -MEM containing 10% FBS, and resuspended in the same medium containing 0.5 mM diisopropylfluorophosphate. The suspension was then put into a plastic cell culture dish (Corning, NY, USA) and cultured at 37°C in 5% carbon dioxide and 95% air for 2 hours. During the culturing, at one hour from the start of culturing, the cell culture dish was replaced with a new one. After the culturing, cells were washed with 10% FBS/ α -MEM three times.

Please replace the paragraph beginning at Page 16, line 5, and ending on Page 16, line 15, with the following:

The culturing was carried out at 37°C in 5% CO₂ - 5% O₂ - 90% N₂ for 4 to 5 days. After culturing cells in each well of the microplate they were washed twice with PBS, and lysed with 180 μ l of 0.2% (w/v) Triton X-100, 1 mM EDTA, 0.12M NaCl, 50 mM HEPES (pH 7.5), and 20 μ l of a substrate, 5.6 mM acetylthiocholine acetylthiocholine iodide, was added thereon. After culturing with shaking at a room temperature for an hour, 20 μ l of the solution was transferred to a microplate for fluorescent assay (Dynatech Micro FLUOR "B" Plate).

Please replace the paragraph beginning at Page 20, line 23, and ending on Page 20, line 37, with the following:

The reaction product was then subjected to second PCR using the primer NI067 and the oligomer 3'-RACE adapter primer included in the 3'-RACE Kit (Gibco BRL) to obtain a DNA fragment of about 900 base pairs. Next, using a direct nucleotide sequence determination method for a PCR product, according to U. Gyllensten et. al., Proc. Natl. Acad. Sci. USA 85: 7652 (1988), the DNA fragment of about 900 base pairs was directly used as a reaction substrate to determine a nucleotide sequence of a portion representing protein and a portion downstream of the protein portion using a Taqu Dye Deoxy Terminator Cycle Sequencing kit available from Applied Biosystem and a fluorescent nucleotide sequencer (Applied Biosystem, Type 370A) according to a manufacturer's manufacturer's instruction. As a result, a sequence from nucleotide number 1081 to 1950 of SEQ ID NO: 30 was shown.

Please replace the paragraph beginning at Page 20, line 38, and ending on Page 21, line 7, with the following:

On the basis of this sequence, oligomer KY100 (SEQ ID NO: 13; corresponding to 1255-1236 of SEQ ID NO: 30) was synthesized. The reaction product obtained by the PCR using NI065 and the oligomer 3'-RACE adapter primer attached to the 3'-RACE Kit (Gibco BRL) was used as a reaction substrate to carry out a further PCR using NI065 and KY100. As a result, a DNA fragment of 807 base ~~paires~~ pairs was obtained.

Please replace the paragraph beginning at Page 21, line 8, and ending on Page 21, line 20, with the following:

This DNA fragment of 807 base paires pairs was directly used as a reaction substrate to determine its nucleotide sequence using Taq Dye Deoxy Terminator Cycle Sequencing kit available from Applied Biosystem and a fluorescent nucleotide sequencer according to ~~a manufacturer's~~ manufacturer's instruction. As a result, a nucleotide sequence from nucleotide number 487 to 1080 of SEQ ID NO: 30 was shown. On the basis of this sequence, oligomers NI073 (SEQ ID NO: 14; corresponding to 864-886 of SEQ ID NO: 30), NI074 (SEQ ID NO: 15; corresponding to 1012 to 992 of SEQ ID NO: 30), and NI075 (SEQ ID No: 16; corresponding to 802-782 of SEQ ID NO: 30) were synthesized.

Please replace the paragraph beginning at Page 36, line 8, and ending on Page 36, line 16, with the following:

This DNA fragment was treated with EcoRI to generate EcoRI cohesive sites at both the ends of the cDNA coding for a megakaryocyte differentiation factor in virtue of EcoRI recognizing sites artificially added to the oligomers NI078 (SEQ ID NO: 32) and NI079 (SEQ ID NO: 33). This cDNA fragment coding for megakaryocyte differentiation factor was introduced into a ~~mammorian~~ mammalian expression vector pdKCR-DHFD at ~~it's~~ its EcoRI recognizing site to obtain pdKCR-DHFR-TPO55.

Please replace the paragraph beginning at Page 36, line 17, and ending on Page 36, line 30, with the following:

The animal cell expression vector pdKCR-dhfr (Oikawa, S. et. al., Biochem. Biophys. Res. Commun. 164, 39, 1989) is a derivative of pKCR (O' Hare et. al., Proc. Natl. Acad. Sci. USA, 78, 1527, 1981) and has SU-40 SV40 early promoter and a rabbit β -globin gene and dhfr (dehydrofolate reductase) gene. Note, a host transformed with the expression vector, was designated as Escherichia coli SBM 308, and deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, 1-3, Higashi 3-chome, Tsukuba-shi, Ibaraki, Japan as FERM P-11506 on June 7, 1990, and transferred to an international deposition under the Budapest treaty as FERM BP-4197 on February 18, 1993.

Please replace the paragraph beginning at Page 36, line 31, and ending on Page 37, line 6, with the following:

The clone pdKCR-DHFR-TPO55 containing megakaryocyte differentiation factor cDNA which was incorporated in-to into pdKCR-DHFR was sequenced using a Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystem) and a fluorescent sequencer (Applied Biosystem Type 370A) according to included instructions. As a result, the determined nucleotide sequence conformed to the sequence of the nucleotide numbers 99 to 1236 of SEQ ID NO: 30 and oligomers NI078 and NI079. In addition, it was confirmed

by the sequencing that a megakaryocyte differentiation factor cDNA inserted into the vector is in correct orientation in relation to an expression vector promoter.

Please replace the paragraph beginning at Page 37, line 7, and ending on Page 37, line 14, with the following:

As shown in the above, once the information of SEQ ID NO: 30 is provided, it is easy for a person skilled in the art that to determine the nucleotide sequence is determined by amplifying cDNA coding for megakaryocyte differentiation factor in total or in a optional portion on megakaryocyte differentiation factor expressing cell line (for example, A431) and boiled cloned in a optional expression vector.

Please replace the paragraph beginning at Page 39, line 14, and ending on Page 39, line 24, with the following:

About 1×10^6 BoMo15A IIc cells were cultured in 4 ml of MGM448 medium containing 10% FBS on the bottom of 25 cm² area of a flask for 2 days by plate culture. To the culture, 0.5 moi of wild type virus B6E or recombinant virus (TPO55-BmNPV) containing a gene coding for megakaryocyte differentiation factor were added and infected, BoMo15A He cells, BoMo15A IIc cells infected, and the cells were cultured at 25°C for 3 days, and total RNA was extracted using Isogen (Wako Pure Chemical). Similarly, total RNA was extracted from non-infected non-infected BoMo 15AIIc cells.

Please replace the paragraph beginning at Page 39, line 25, and ending on Page 40, line 5, with the following:

Next, 1 ~~Mg~~ mg of the RNA thus extracted was size-fractionated by agarose gel electrophoresis, and the separated RNA was transferred to a Zetaprobe ~~membrane~~ membrane by the capillary action. The ~~membrane~~ membrane was soaked in a hybridization buffer containing megakaryocyte differentiation factor cDNA (:PCR product amplified with KY100 and NIO65 described in Example 2.1) (TPO55 probe DNA) labeled with digoxigenin (Boehringer Mannheim), and the mixture was incubated at 42°C for 12 hours to allow the formation of specific complex of recombinant megakaryocyte differentiation factor mRNA and the TPO55 probe DNA thereof. The complex was then reacted with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim), and the complexed megakaryocyte differentiation factor mRNA was ~~detected~~ detected by chemoluminescence generated by hydrolysis of Lumigen PPD (AMPPD) (Boehringer Mannheim) according to manufacture's instructions with alkaline phosphates.

Please replace the paragraph beginning at Page 40, line 38, and ending on Page 41, line 11, with the following:

50 ml of the hemolymph of the silkworms obtained in the above section (5) was thoroughly dialyzed against a 20 mM Tris/HCl (pH 7.4) buffer, and applied to a Matrex Blue A column (φ2.5 × 15 cm) equilibrated with the same buffer. The column was thoroughly washed with the same buffer to eliminate ~~a~~ an unbound fraction, and a bound

protein was eluted by a concentration gradient of 0 to 1M NaCl. An elution profile for megakaryocyte differentiation activity of the hemolymph obtained from silkworms injected with the recombinant virus was compared with that for a wild type virus.

Please replace the paragraph beginning at Page 41, line 17, and ending on Page 41, line 29, with the following:

Although Bombyx mori vaeurovirus baculovirus transfer vector pBm4, Bombyx mori nuclear polyhedrosis virus PbE and Bombyx mori cells BoMo15AIIC were used in Example 4, the present invention is not limited to the use of these materials. Namely, other baculovirus transfer vector (such as pBK283, pBKblue, available from Funakoshi) Bombyx mori nuclear polyhedrosis virus (such as purified DNA available from Funakoshi), Bombyx mori cells (such as BmN4 cells, available from Funakoshi) can be easily used by a person with ordinary skill in the art to obtain a megakaryocyte differentiation factor.